Impurity fingerprints for the identification of counterfeit medicines - a feasibility study

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Abstract

Most of the counterfeit medicines are manufactured in non good manufacturing practices (GMP) conditions by uncontrolled or street laboratories. Their chemical composition and purity of raw materials may, therefore, change in the course of time. The public health problem of counterfeit drugs is mostly due to this qualitative and quantitative variability in their formulation and impurity profiles.

In this study, impurity profiles were treated like fingerprints representing the quality of the samples. A total of 73 samples of counterfeit and imitations of Viagra® and 44 samples of counterfeit and imitations of Cialis® were analysed on a HPLC-UV system. A clear distinction has been obtained between genuine and illegal tablets by the mean of a discriminant partial least squares analysis of the log transformed chromatograms. Following exploratory analysis of the data, two classification algorithms were applied and compared. In our study, the k-nearest neighbour classifier offered the best performance in terms of correct classification rate obtained with cross-validation and during external validation. For Viagra®, both cross-validation and external validation sets returned a 100% correct classification rate. For Cialis® 92.3% and 100% correct classification rates were obtained from cross-validation and external validation, respectively.

Keywords:
Impurities, Fingerprints, Classification, Counterfeit, Phosphodiesterase type 5 inhibitors

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1. Introduction

The problem of pharmaceutical counterfeiting has widely grown since 1990 when it has been first detected. In industrialized countries, drug counterfeiting accounts for less than 1% of the market value, but in developing countries in Africa, Asia and Latin America, it represents a much higher percentage of the medicines on sale mostly due to the lack of regulatory systems and effective market control. The World Health Organization (WHO) defines a counterfeit medicine as “one which is deliberately and fraudulently mislabelled with respect to identity and/or source” [1, 2]. This definition underlines the fact that the source and the manufacturing conditions of the counterfeit drugs are unknown. Most of them are manufactured in non good manufacturing practices (GMP) environment by uncontrolled or street laboratories [3]. Thus, their chemical composition and purity of raw materials may change in the course of time and they may not meet the European Pharmacopoeia requirements. The risks for public health of counterfeit drugs are mostly due to this variability. The impurities in drug products may have different origins [4], for instance:

- starting materials,
- by-products and residual solvents from the API synthesis,
- degradants formed during the process and long-term storage, and
- contaminants from packaging components and other drug products manufactured in the same facility.

The impurities can also be formed as a result of heat, light and oxidants. They can be catalysed by trace metal impurities, change in the pH of the formulation, interaction with packaging components [5], excipients and other active ingredients in the case of combination products.

Several studies have reported the use of spectroscopic fingerprints provided by nuclear magnetic resonance (NMR) [6-8], near infrared spectroscopy (NIR) [9-11] and Fourier transform infrared spectroscopy (FT-IR) [12, 13] in the field of quality control and/or discrimination of food origin. The main drawback of these techniques is their complexity of interpretation. This is why, more and more, chromatographic techniques are used to provide fingerprints. The use of HPLC-UV by Dumarey et al. allowed the synthesis pathway of four acetaminophen formulations to be distinguished [14]. The HPLC-DAD fingerprints have also been used in the field of quality control [15-17], but to obtain the maximum information from the samples, the use of a coupled mass spectrometer is necessary.

LC-MS has been used for impurity profiling [18, 19] and is widely spread for the control of herbal medicines [20-23]. Although less used, the fingerprinting by GC-MS has also gained attention [24-26].

In this study, 73 counterfeit and imitations of Viagra®, 10 genuine Viagra®, 44 counterfeit and imitations of Cialis® and 5 genuine Cialis® were analysed. The aim of this study was to discriminate illegal samples from genuine ones based on their impurity profiles and to build a predictive classification model. For this purpose, k-nearest neighbour (k-NN) and soft independent modelling by class analogy (SIMCA) were applied and compared.

2. Experimental

2.1. Samples

The counterfeit and imitation tablets were donated by the Federal Agency for Medicines and Health Products in Belgium (AFMPS/FAGG). They all come from postal packs ordered by individuals through internet sites. All samples were delivered in blisters or in closed jars with or without packaging. All samples, once received, were stored at ambient temperature and protected from light.

Pfizer SA/NV (Belgium) kindly provided one batch of each different dosage of Viagra® (25mg, 50mg, 100mg). Two other batches of each dosage were purchased in a local pharmacy in Belgium.

Eli Lilly SA/NV (Benelux) kindly provided one batch of commercial packaging of Cialis® (10mg and 20mg). Two other batches of Cialis® 20mg were purchased in a local pharmacy in Belgium.

All references were delivered in closed blisters with packaging and were stored protected from light at ambient temperature.

2.2. Instrumental

Impurity profile analyses were performed on an Alliance 2690 HPLC system (Waters, Milford, USA) coupled to a 2487 dual λ absorbance detector (Waters). Data acquisition and treatment were performed with the Empower2 software (Waters).

For the Viagra-like samples, the HPLC method is the one published in Pharmeuropa (draft monography for the European Pharmacopoea) [27]. Chromatography was performed at 30°C on a Symmetry C_{18} 150 mm x 4.6 mm with a 5µm particle size (Waters). The mobile phase was a mixture of 17 volumes of acetonitrile, 25 volumes of methanol and 58 volumes of a 0.7 % (v/v) solution of triethylamine adjusted to pH 3.0 ± 0.1 with orthophosphoric acid. The flow rate was set at 1 mL min^{-1} and the injection volume was 20 µl of a solution of 500 µg mL^{-1} of sildenafil. The samples were diluted in the mobile phase and the detection was performed at 290 nm (λ_{max} for sildenafil).

For the Cialis-like samples, the method has been slightly adapted from the Pharmeuropa method [28]. Chromatography was performed at 30℃ on a Zorbax C_8 150 mm x 4.6 mm with a 3,5µm particle size (Agilent Technologies, Santa Clara, USA). The mobile phase was a mixture of 35 volumes of acetonitrile and 65 volumes of a 0.1 % (v/v) aqueous solution of trifluoroacetic acid. The flow rate was set at 1 mL min^{-1} and the injection volume was 20 µl of a solution of 400 µg mL^{-1} of tadalafil. The samples were diluted in the mobile phase and the detection was performed at 285 nm (λ_{max} for tadalafil).

2.2. Data analysis

All data treatments were done using Matlab (The Matworks, Natick, MA, USA, version 7.9.0). The SIMCA analysis was performed using the PLS_toolbox for Matlab (Eigenvector Research, Inc., Wenatchee, WA, USA, version 6.0.1).

2.3. Chemometric methods

2.3.1. Data pre-processing

Pre-processing of chromatographic fingerprints is a crucial step that can strongly affect results of further analysis and interpretation. Usually, pre-processing of chromatographic fingerprints requires improvement of the signal-to-noise ratio (noise reduction, baseline elimination), normalization (e.g., to remove differences in sample volumes) and peak alignment [29]. In our study, noise reduction and baseline correction were unnecessary because all signals have a relatively good signal-to-noise ratio. A detailed analysis of consecutive signals revealed a systematic offset. Therefore, from all elements of the signals at a given retention time their minimal value was subtracted.

In our study only a simple peak correction was needed to obtain a satisfactory overall alignment of all signals. The major steps of the alignment procedure followed by us can be summarized as follows. Firstly, the target signal was selected as the one that resembles the largest mean correlation between all chromatograms [30]. Then, in all signals the largest peak was identified (using the threshold approach) and this peak served as a marker peak for the alignment. Afterwards, all signals were transformed using the linear interpolation so that positions of the largest peak in all signals matched the position of the largest peak in the target signal. During alignment procedure, each signal was split into two parts defined by the apex of the marker peak and then independently warped using linear interpolation towards the corresponding section in the target signal.

The linear interpolation is a procedure of compressing or extending a signal by changing a sampling rate over x-axis of a signal. Bearing in mind the desired number of sampling points of a signal, a new uniform grid of points is constructed over a certain range (with less or more points). Then, the unknown y-values for x-points on a new grid of points are calculated using the following expression:

$$y = y_0 + (x - x_0)\frac{y_1 - y_0}{x_1 - x_0} \quad (1)$$

where, y is the predicted y-value at location x on a new grid of points, x_0 and x_1 are x-points of the original axis and define interval containing point x, whereas y_0 and y_1 are their respective y-values.

2.3.2. Principal component analysis

Principal component analysis (PCA) is an exploratory technique. It allows original variables, **X**, to be represented by a limited number of orthogonal latent variables, called principal components (PCs). They are constructed as linear combinations of original variables and explain the largest part of the data variability [31]. The importance of the original variables in the construction of a given principal component is indicated by the magnitude of its absolute loading value. The projections of the objects onto the space of principal components are called the scores of the objects. The selected pairs of scores are summarizing a large part of the data variance. They can be further used to examine and interpret similarities among samples. More details about the PCA method can be found in [32].

2.3.3. Discriminant partial least squares

Discriminant partial least squares (D-PLS) is a supervised method, aiming to discriminate among known groups of samples. This goal is achieved using a few latent variables, called the PLS-factors being linear combinations of the original variables, **X**. They are constructed in such way that the covariance between the data variable and the response variable, **y**, which represents to which group a sample belongs, is maximal. This is why the PLS-factors capture better differences among known groups of samples than PCA latent factors. Especially when groups of samples are not distributed along the directions of the largest data variability. In this study, a binary response variable was used to code two groups of samples. Zeros in the response variable represent illegal samples and ones genuine samples. This

differentiation between two groups of samples was possible since the genuinity of the reference samples is certified [32].

2.3.4. Selection of a test set for external validation of models

In order to perform an external validation of the classification models, the aligned chromatographic fingerprints were split into a training and test set applying the Kennard and Stone algorithm [33]. The Kennard and Stone algorithm allows the selection of samples in the dataset in such way that their distribution in the data space is as uniform as possible. The uniform selection of samples guarantees that the main sources of data variability will be incorporated during the construction of a model. The model will thus be general enough when used for prediction purposes.

2.3.5. K-nearest neighbour

The k-nearest neighbour method [34], k-NN, is a classification technique where neighbourhoods of training set objects are used for the construction of classification rules. During the classification procedure, the Euclidian distances between an unknown object and each of the objects of the training set are computed. For a dataset with n samples, n distances are calculated. Then, for a new object its k closest neighbours from the training set are examined. The unknown object is classified into the group to which the majority of the k neighbouring objects of the training samples belong.

2.3.6. Soft Independent Modelling by Class Analogy (SIMCA)

SIMCA [34] is a classification technique that models each group of samples separately using a few principal components obtained from PCA. The optimal number of principal components, required to describe the training class, is evaluated using a cross-validation procedure. To construct classification rules, two critical values are considered obtained for the Euclidian distances towards the SIMCA model (the so-called orthogonal distances) and the Mahalanobis distances computed in the space of scores. The two critical values define a limited space around the samples of the training set in the model space with respect to their orthogonal distance and Mahalanobis distance. The position of a new object in the studied space is computed using the scores and loadings of the constructed PCA model. If the object is located within the defined limited space of orthogonal and Mahalanobis distances for the training class then it is said to belong to this class. Otherwise, the object is considered as an outlier, i.e. not belonging to this group. Confidence limits were set at 95%. Since SIMCA

belongs to the so-called soft classification methods, it is possible that a new sample can be assigned to one or more existing groups or to any. This is a direct consequence of building disjoint classification models for each group of samples.

3. Results and Discussion

3.1. Measurements

Each sample has been analysed in triplicate. Figure 1 shows typical impurity profiles of both legal and illegal tablets. The chromatograms were aligned using linear interpolation with respect to the highest peak. Figure 2 shows a signal of the chromatograms before and after alignment. After alignment, replicate chromatograms obtained for a given sample were averaged.

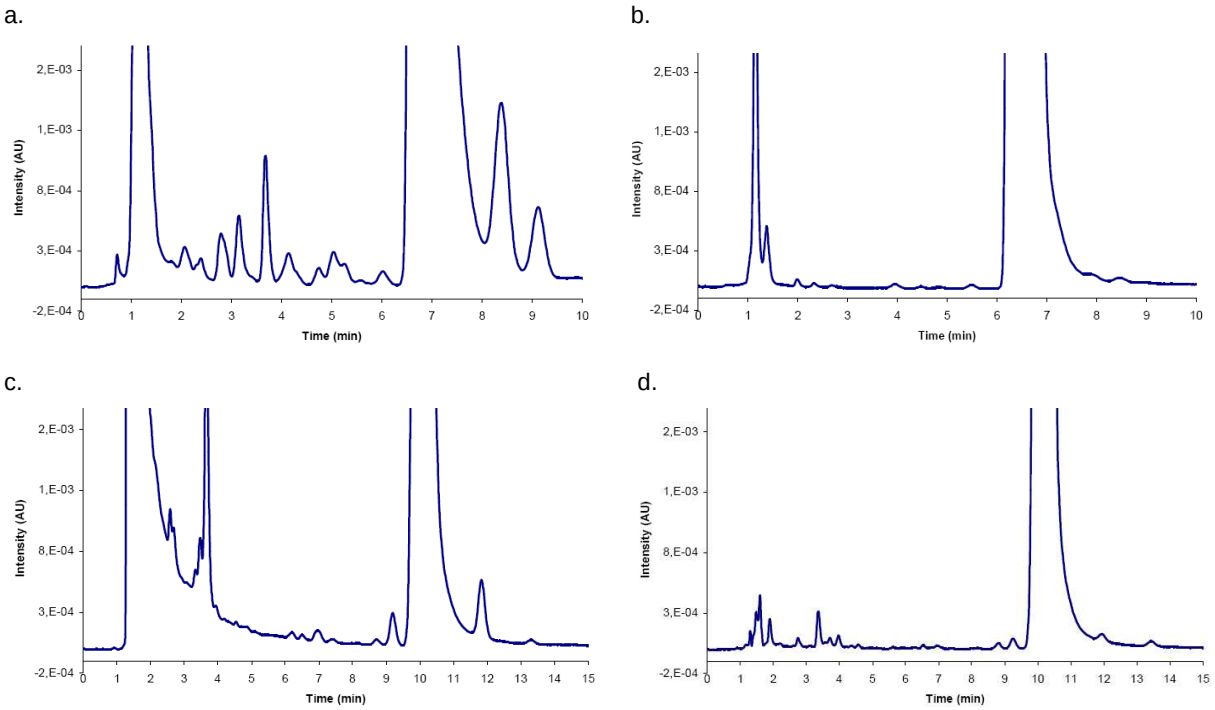


Figure 1:

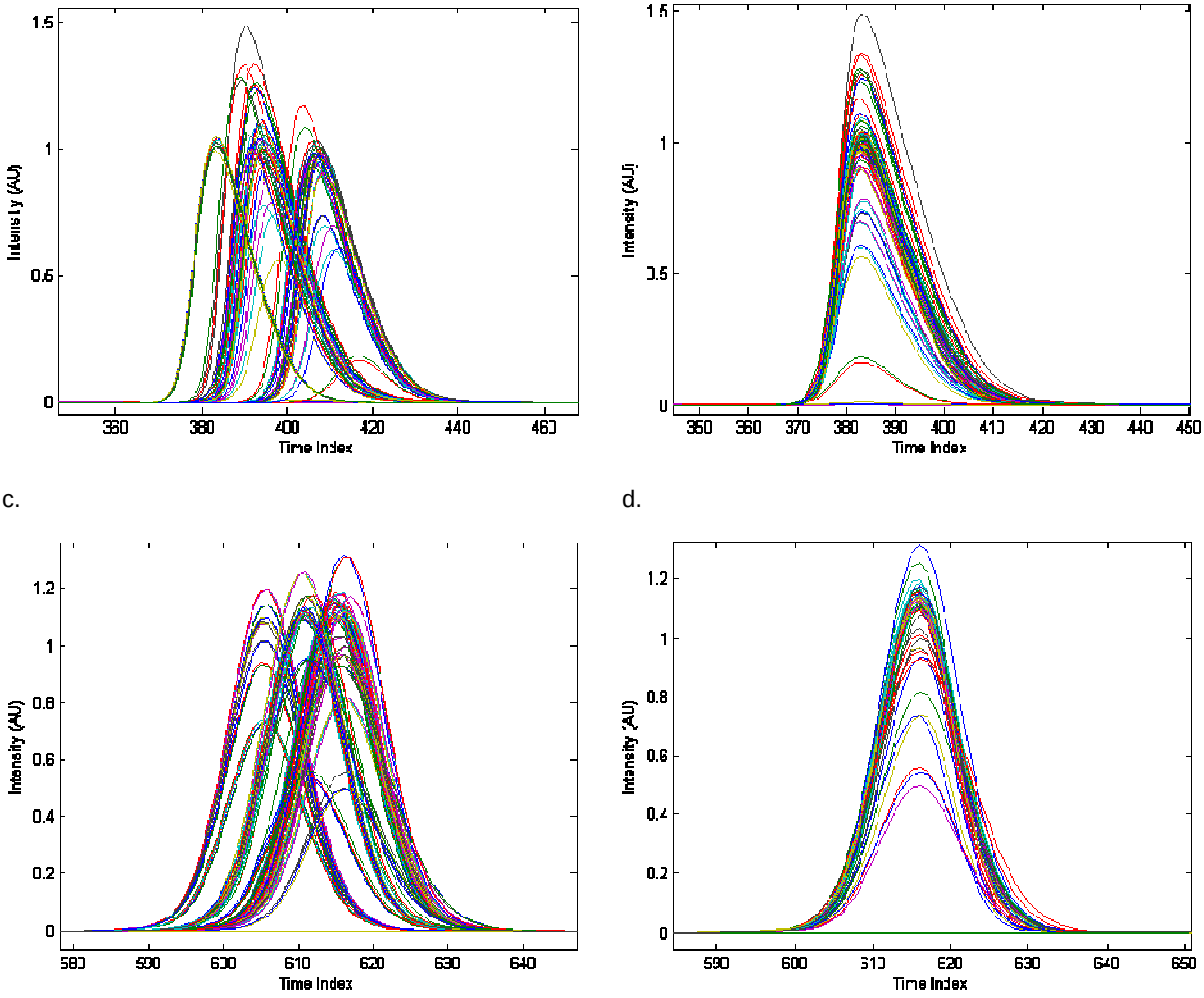
a. Impurity profile of a counterfeit tablet of Viagra®
b. Impurity profile of a genuine tablet of Viagra®
c. Impurity profile of a coloured imitation tablet of Cialis®
d. Impurity profile of a genuine tablet of Cialis®.

3.2. Exploratory analysis on raw data

An exploratory analysis of raw chromatographic fingerprints using both PCA and D-PLS did not reveal a clear discrimination between genuine and illegal samples. This is mainly due to

the fact that too much importance was given to noise and inevitable baseline shifts. In order to reduce the importance of these differences, a common logarithmic transformation of the dataset was performed.

a. b.



c. d.

Figure 2:

a. Superposition of the non aligned peak of sildenafil
b. Superposition of the aligned peak of sildenafil;
c. Superposition of the non aligned peak of tadalafil
d. Superposition of the aligned peak of tadalafil.

3.3. Viagra®

3.3.1. Exploratory analysis

3.3.1.1. PCA analysis

The log transformed data, containing fingerprints of Viagra® samples, have been compressed using PCA into a few principal components to reveal possible differences between genuine and illegal samples. Unfortunately, the discrimination obtained is too weak to be acceptable (Figure 3a). Therefore, the supervised D-PLS approach was applied to enhance this discrimination.

a. b.

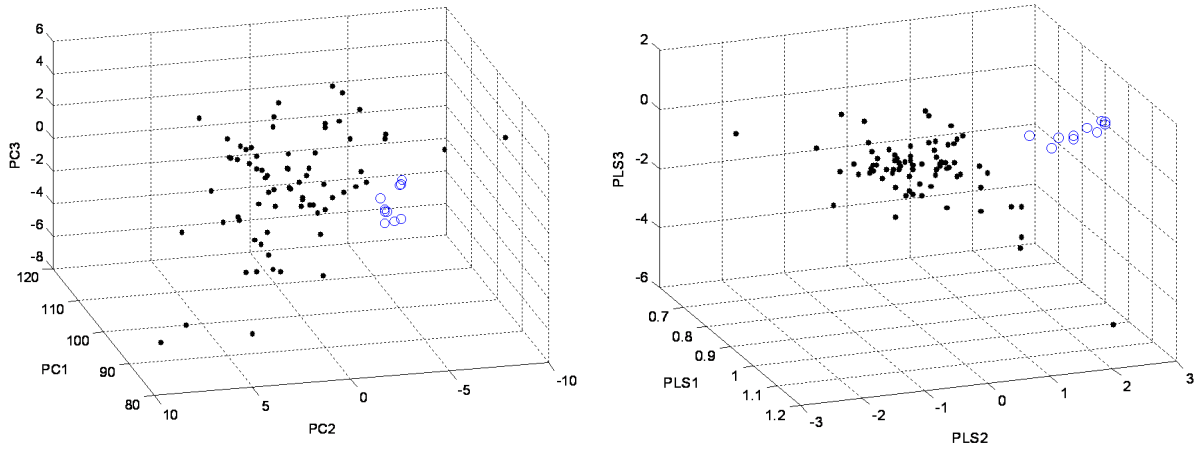


Figure 3:
a. PCA 3 dimensional plot of the log transformed Viagra® dataset. Black points are the illegal samples and blue circles are the genuine ones;
b. PLS 3 dimensional plot of the log transformed Viagra® dataset. Black points are the illegal samples and blue circles are the genuine ones.

3.3.1.2. D-PLS analysis

Figure 3b gives an indication that there exists a good discrimination between genuine and illegal samples.
The illegal samples are spread widely and no clustering tendency among them was observed.

3.3.1.3. Classification

The possibility to predict whether a sample is genuine or not has been tested using two different classification algorithms: k-NN and SIMCA. Before construction of classification

models, the log transformed fingerprints were divided into a training set and a test set using the Kennard and Stone algorithm. For each classification model, its prediction abilities were examined by means of internal (using leave-one-out cross-validation) and external validation.

For classification purposes, training and test sets were constructed such that the training set contained 55 illegal samples and eight genuine samples, and the test set 20 samples including two genuine samples. Figure 4 provides a confirmation of the relatively homogenous distribution of the test set among the samples of the training set.
For the studied data, the k-NN classifier allows 100% of correct classification to be reached evaluated using leave-one-out cross-validation and external validation. The closest neighbours were equal to 3.

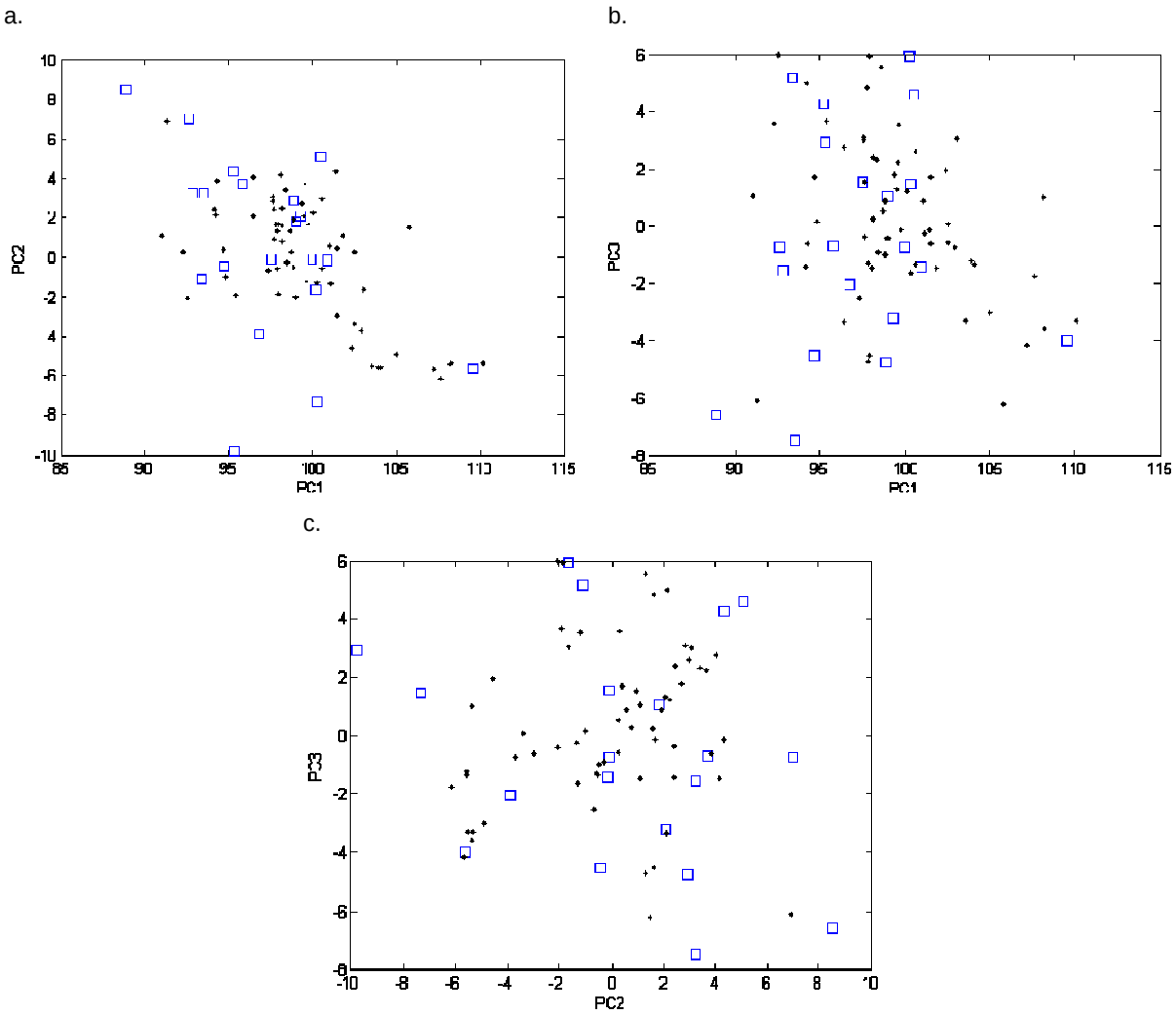


Figure 4: a. PC1-PC2; b. PC1-PC3 and c. PC2-PC3 plot of the training set (black points) and the test set (blue squares) determined by the Kennard and Stone algorithm applied on the Viagra® dataset.

When the SIMCA classification has been applied, both legal and illegal groups of samples were described by one principal component. With the SIMCA model tested using cross-validation and external validation set 100% correct classification rates were achieved.
The comparison of the two classification methods indicates that k-NN is the best suited method for this dataset because of the satisfactory results obtained and its simplicity (Table 1).

Table 1: Results obtained applying classification algorithms.

Viagra-like samples		TP	FP	TN	FN	% CCR
k-NN						
	Training set	8	0	55	0	100
	Test set	2	0	18	0	100
SIMCA						
	Training set	8	0	55	0	100
	Test set	2	0	18	0	100
Cialis-like samples		TP	FP	TN	FN	% CCR
k-NN						
	Training set	3	3	33	0	92,3
	Test set	2	0	8	0	100
SIMCA conditions 1						
	Training set	3	5	31	0	87,2
	Test set	2	0	8	0	100
SIMCA conditions 2						
	Training set	3	3	33	0	92,3
	Test set	2	1	7	0	90

TP, true positives (genuine correctly classified); FP, false positives (illegal classified as genuine); TN, true negatives (illegal correctly classified); FN, false negatives (genuine classified as illegal); CCR, correct classification rate.
See text for SIMCA conditions 1 and 2.

3.4. *Cialis®*

3.4.1. *Exploratory analysis*

3.4.1.1. PCA analysis

For the Cialis® set of samples, the exploratory analysis did not highlight evident differences between genuine and imitation samples. In general, the genuine samples were scattered among professional imitation samples. The characteristics of the professional imitations are that they contain the correct API within 90-110% of the declared value and that they do not have the same appearance as genuine tablets.

They can be considered as good quality samples in comparison with the other illegal samples. It is therefore comprehensible that the difference between the professional imitations chromatograms and the genuine ones are not directly related to main data variability and thus the first principal components did not reveal a good discrimination between the two groups of samples. For this reason again, D-PLS was applied.

3.4.1.2. D-PLS analysis

As can be seen in Fig. 5, discrimination between genuine and illegal samples was achieved. The discrimination is mainly captured by PLS factor 2 and 3. Although the illegal samples seem quite close to each other, some differences may be observed. A group of four samples can be distinguished from the other ones (samples in a circle). These samples represent non-coated tablets, and their cores have intensive orange colour. Chromatographic fingerprints of these tablets reveal an intensive absorbance of the UV light at signal's beginning. In Fig. 5, one can observe that one sample, indicated with an arrow, is relatively far away from remaining illegal samples. This phenomenon can be explained by the fact that this sample contains only traces of both sildenafil and tadalafil.

In Fig. 5c, a separation line is drawn that splits the illegal samples in two groups, denoted as A and B. Group A (above the discrimination line) may be considered as a group containing samples of bad quality and/or recognized as dangerous. Indeed, 13 samples out of 17 contain, besides tadalafil, sildenafil in amounts as high as 7mg per tablet. Among these samples were all the counterfeited Cialis® tablets. On the other hand, group B (below the separation line) contains imitations samples of reasonable good quality. These tablets contain only tadalafil (except two of them, in which traces of sildenafil were identified) within the 90-110% range of the declared value. It can be concluded that samples from group A are more hazardous than samples from group B because their chemical composition seems

whimsical. This lack of confidence in the chemical composition of the medicines increases the hazard of their intake.

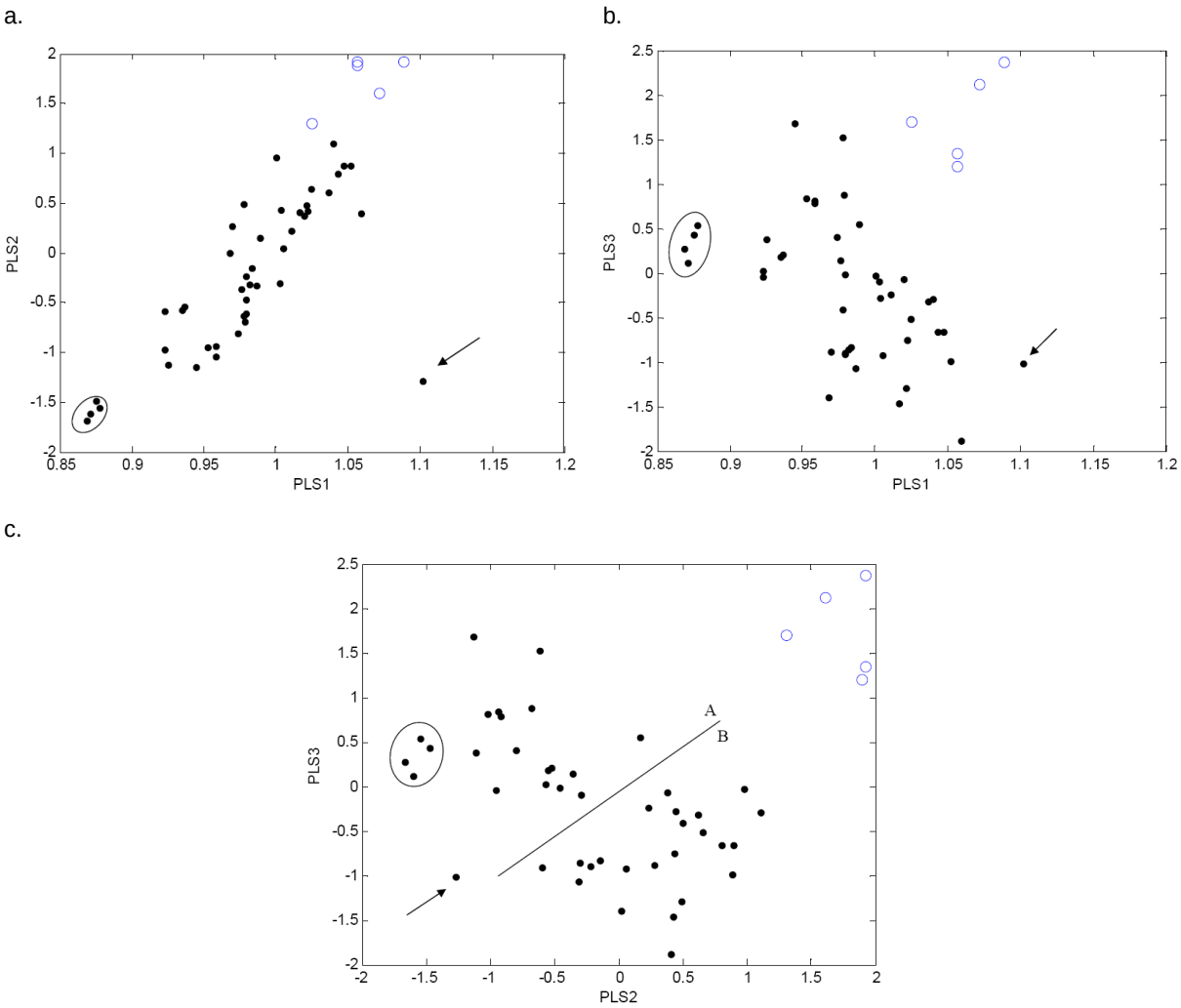


Figure 5:

a. PLS1-PLS2 plot of the log transformed Cialis® dataset. Black points are the illegal samples and blue circles are the genuine ones. For explanations about the arrow and the circle see text section 2.3.2.
b. PLS1-PLS3 plot of the log transformed Cialis® dataset. Black points are the illegal samples and blue circles are the genuine ones. For explanations about the arrow and the circle see text section 2.3.2.
c. PLS2-PLS3 plot of the log transformed Cialis® dataset. Black points are the illegal samples and blue circles are the genuine ones. All samples above the line (except genuine ones and the ones that are surrounded) form the Group A. The samples under the line form the Group B. For explanations about the arrow and the circle see text section 2.3.2.

3.4.1.3. Classification

By analogy with the Viagra® samples, k-NN and SIMCA were applied on the Cialis® dataset. To construct and validate the classification models, the data was split into a training set containing 36 illegal samples and three genuine samples and a test set of ten samples including two genuine samples. The training and test set were determined using the Kennard

and Stone algorithm. Figure 6 shows a quite homogenous distribution of the test set samples among the samples of the training set.

The k-nearest neighbour classifier allows to obtain 92.3% correct classification rate (3 illegal samples misclassified, and all genuine samples were correctly classified) evaluated using leave-one-out cross-validation. All samples from the independent test set were classified appropriately. The closest neighbours were equal to 3.

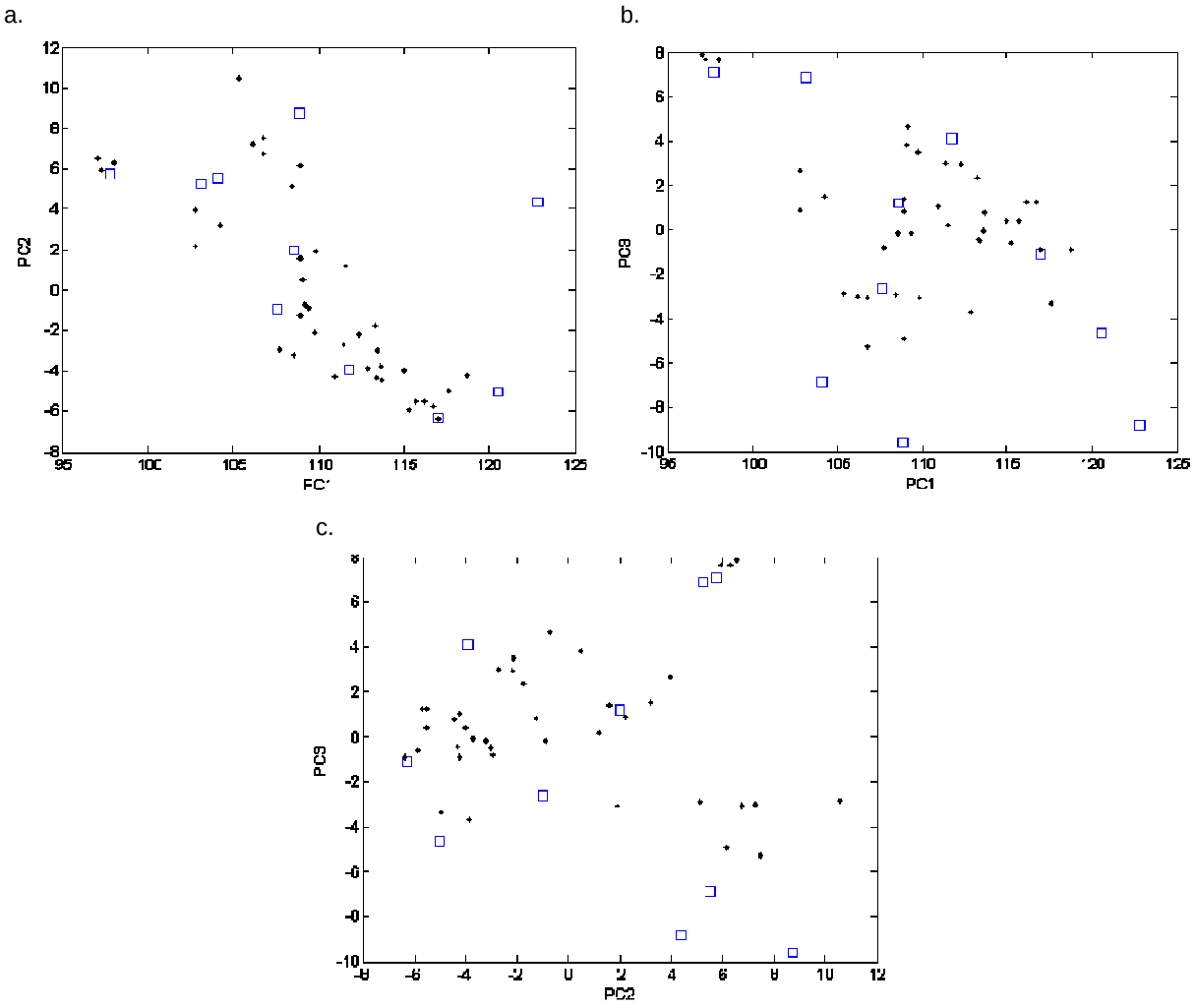


Figure 6:

a. PC1-PC2 plot of the training set (black points) and the test set (blue squares) determined by the Kennard and Stone algorithm applied on the Cialis® dataset.
b. PC1-PC3 plot of the training set (black points) and the test set (blue squares) determined by the Kennard and Stone algorithm applied on the Cialis® dataset.
c. PC2-PC3 plot of the training set (black points) and the test set (blue squares) determined by the Kennard and Stone algorithm applied on the Cialis® dataset.

In addition to k-NN classification, the SIMCA classification was also evaluated. One principal component was found to be sufficient to build the model for the genuine samples. To describe the illegal samples, models containing 2 and 3 PCs were tested. When using 2 PCs (conditions 1) to model the illegal samples, a correct classification rate of 87.2% was

achieved (5 illegal samples were misclassified, and all genuine samples were correctly classified) during cross-validation. External validation resulted in a 100% correct classification rate. When 3 PCs (conditions 2) were used to model the illegal samples, cross validation returned 92.3% correct classification rate (3 illegal samples misclassified, all genuine samples correctly classified) and external validation misclassified 1 sample out of ten, though all genuine samples were classified as genuine.

The comparison of the two classification approaches favours k-NN since it allows obtaining a slightly better classification results compared to SIMCA (Table 1). It can be finally concluded that k-NN is the best suited method since it provides satisfactory classification results and is simple to implement.

4. Conclusion

As the manufacturing processes of counterfeit drugs are not controlled properly or made by not scrupulous people, their chemical composition is not known. This problem ranges from using wrong excipients to instable or improper quantity of active pharmaceutical ingredient. Even when the correct amount of API is present at the correct dosage, impurities may be detected at too high concentrations. These impurities come from starting materials of questionable quality, cross-contamination, improper manufacturing processes and/or conservation conditions. The presence of various compounds in various amounts represents a real thread for the public health.

In this study, it has been investigated whether differences in impurity profiles could be used to detect counterfeit tablets. Impurity profiles of 77 counterfeit and imitations of Viagra®, 10 genuine Viagra®, 44 counterfeit and imitations of Cialis® and 5 genuine Cialis® were obtained by applying classical reversed phase methods on a common HPLC-UV system.

After the log transformation of chromatographic fingerprints and using the discriminant PLS approach for exploratory analysis, a clear discrimination between legal and illegal samples was achieved.

Two classification algorithms (k-NN and SIMCA) were applied and compared in terms of the correct classification rates obtained during leave-one-out cross-validation and for external validation samples. From the obtained results, it is concluded that the k-NN algorithm is the best suited since it provides 100% correct classification rate for both cross-validation and external validation for the Viagra® dataset and 92.3% (all genuine samples correctly classified) and 100% correct classification rate during respectively cross-validation and external validation for the Cialis® dataset.

To the best of our knowledge, this is the first time that chromatographic fingerprints are used to distinguish counterfeit medicines according to their impurities content. The obtained results show that this would be interesting to develop a routine method to obtain impurity profiles that could be used to detect counterfeit tablets by a control laboratory. This might be found as a useful approach especially in developing countries with basic analytical equipment.
However, further investigations should be done to see whether the obtained results may be applicable to other kind of medicines.

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